

**HONORS THESIS ABSTRACT
THESIS SUBMISSION FROM**

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Thesis Title: Cytoskeletal Coordination of Pancreatic Function

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Advisor's Dept: Biological Sciences

Discipline: Cell Physiology

Year: 2000

Page Length: 36

Bibliography: Included

Illustrated: Yes

Published: No

Copies Available: Hard Copy

Abstract

The pancreas gland is composed of both exocrine and endocrine components. The endocrine component regulates blood sugar levels and the exocrine component produces digestive enzymes that are secreted into the small intestine to aid in digestion. The pancreas must balance the production of new enzymes with the release of active enzymes. Enzyme production begins in the ER and undergoes modification in the golgi apparatus where inactive enzymes are packaged in vesicles. The vesicles dock with the plasma membrane and are secreted into the small intestine after food triggers the release of activating hormones. Although these mechanisms outlining both production and release are known, coordination between the two processes remains undetermined.

The present project was developed on the hypothesis that the cytoskeleton contributes to the coordination between enzyme production and release. The cytoskeleton has historically been associated with maintaining cell structure and facilitating movement; however, recent studies have shown cytoskeletal elements attached to nonstructural proteins and signaling molecules. The present results show that ERK phosphorylation decreases when microtubules are depolymerized with colchicine. However, inhibition of myosin ATPase activity by 2,3-butanedione monoxime (BDM) causes an increase in ERK phosphorylation and a decrease in secretion. Data with the myosin motor protein inhibitor ML-9 also showed an increase in ERK phosphorylation but a decrease when intracellular calcium was increased. The results support the requirement for the cytoskeleton system in pancreatic signalling and that the microfilaments are necessary for the coordination of production and exocytotic release.

Northern Illinois University

Cytoskeletal Coordination of Pancreatic Function

**A Thesis Submitted to the University Honors Program
In Partial Fulfillment of the
Requirements of the Baccalaureate Degree
With University Honors**

Department Of Biological Sciences

**By
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**DeKalb, Illinois
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ABSTRACT

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The present project was developed on the hypothesis that the cytoskeleton contributes to the coordination between enzyme production and release. The cytoskeleton has historically been associated with maintaining cell structure and facilitating movement; however, recent studies have shown cytoskeletal elements attached to nonstructural proteins and signaling molecules. The present results show that ERK phosphorylation decreases when microtubules are depolymerized with colchicine. However, inhibition of myosin ATPase activity by 2,3-butanedione monoxime (BDM) causes an increase in ERK phosphorylation and a decrease in secretion. Data with the myosin motor protein inhibitor ML-9 also showed an increase in ERK phosphorylation but a decrease when intracellular calcium was increased. The results support the requirement for the cytoskeleton system in pancreatic signalling and that the microfilaments are necessary for the coordination of production and exocytotic release.

ACKNOWLEDGEMENTS

I wish to express my appreciation toward the University Committee on Undergraduate Research and Artistry whose undergraduate award made this research possible. Their continued support of student body endeavors has allowed me to successfully conduct independent laboratory research and produce this final undergraduate thesis. I also wish to thank the University Honors Program and Biological Science Department for their support and guidance throughout my undergraduate career. Finally, I would like to recognize the efforts and counsel of Rhykka Panozzo and especially Dr. Kenneth Gasser, whose teachings will forever guide me through my life.

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INTRODUCTION

Multicellular organisms sustain function through complex biochemical pathways that coordinate multiple interrelated cellular events. Detailed steps outlining the sequential activity of many biochemical pathways have been identified scientifically, however the means of coordination between simultaneous events remains undetermined. The mammalian pancreas presents such a model for cellular coordination. To ensure enzyme secretion, the pancreas must balance the production of new enzymes with the release of active enzymes. Individually, regulation of enzyme release and production has been determined, but coordination between the two is unclear.

The pancreas is a digestive gland that contains both exocrine and endocrine components. The endocrine component releases the hormones insulin, glucagon, and somatostatin into the blood stream to regulate blood sugar levels. The exocrine component produces and releases digestive enzymes and alkaline buffers to aid in digestion. The exocrine cells are made up of acinar cells and ductal epithelial cells. Combined, these cells release an alkaline fluid secretion into the duodenum through the pancreatic duct. The fluid secretion contains an assortment of digestive enzymes that hydrolyze ingested macromolecules into subunits the body can then absorb.

Secretions of the exocrine pancreas are regulated by hormones released by the duodenum. As acidic chyme passes from the stomach to the small intestine, the duodenum releases the hormones secretin and cholecystokinin (CCK). Secretin stimulates the release of an alkaline fluid which neutralizes the acidic chyme.

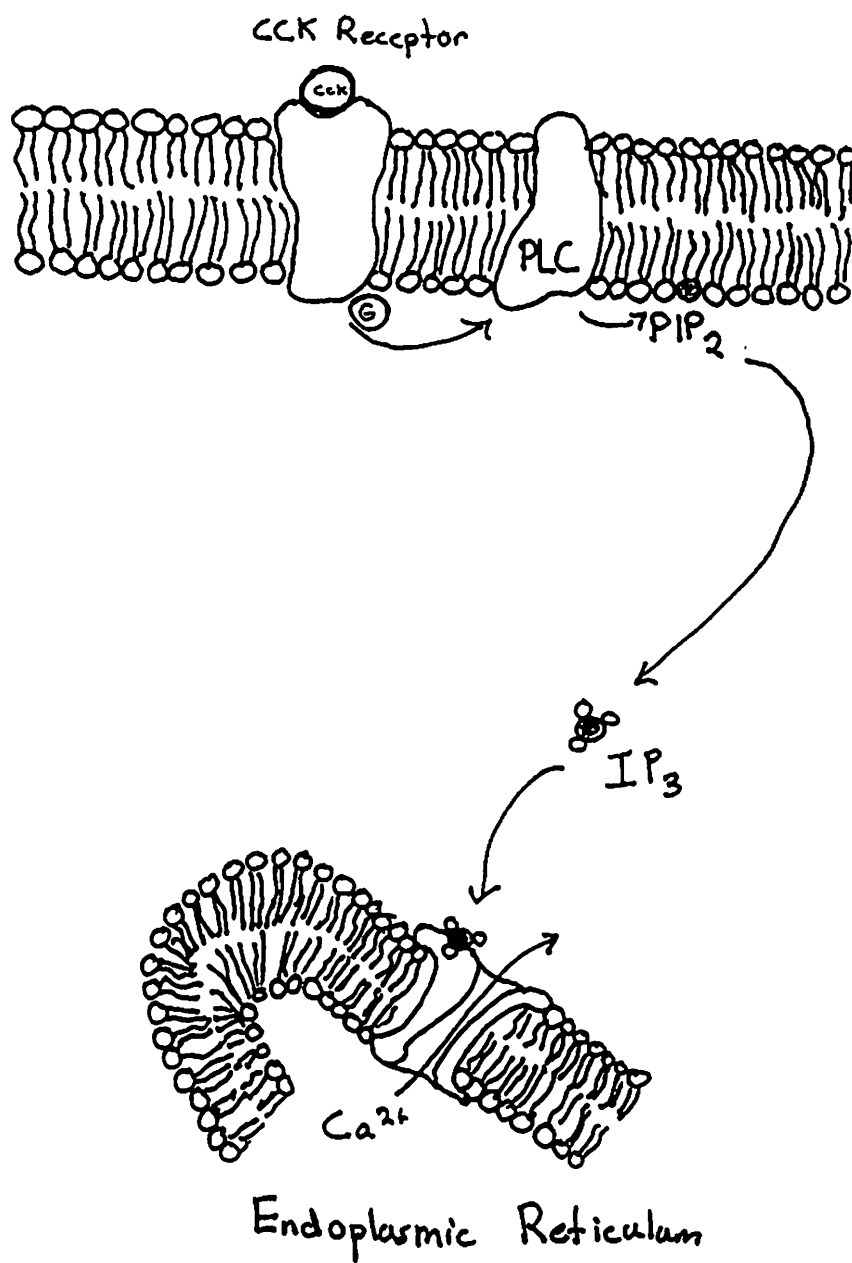
Furthermore, digestive enzymes are denatured by acid and therefore function better in

the slightly alkaline conditions. CCK is the primary hormone responsible for stimulating the pancreas to produce and release the digestive enzymes from acinar cells.

In greater detail, CCK stimulates acinar cells through a G-protein-coupled receptor to increase intracellular calcium and elicit amylase secretion (Torgerson and McNiven, 1999). As CCK binds to an extracellular plasma membrane receptor, the signal is transduced by a guanine nucleotide-binding protein (G-Protein). These transducers transmit an external stimuli to an internal cytoplasmic enzyme. When CCK binds to its receptor, a conformational change occurs within the receptor which subsequently activates the G-protein, Gq, located on the cytoplasmic side of the plasma membrane. The inactive form of Gq is bound to GDP and when CCK binds to its receptor, Gq releases GDP and binds GTP. The α -subunit of the Gq-G-protein dissociates from the Gq complex after GTP binds. The subunit is then free to move along the plasma membrane until it encounters and activates the effector enzyme phospholipase C (PLC). Also located on the cytoplasmic side of the membrane, PLC hydrolyses the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Both IP_3 and DAG act as second messengers transmitting the message deep into the acinar cell.

Once released, IP_3 travels through the cytoplasm until it binds to a calcium channel located on the surface of the endoplasmic reticulum (ER). IP_3 binding causes the calcium channels to open. Calcium stored within the ER lumen diffuses out of the calcium channels and increases cytosolic calcium concentration (Figure 1). The

Figure 1. A model of CCK stimulating the CCK receptor resulting in binding of IP₃ to the calcium channels located on the ER. CCK binds to a G-protein-coupled receptor promoting Gq activation. Gq α binds GTP and activates PLC. PLC hydrolyses PIP₂, yielding IP₃ and DAG. IP₃ travels through the cytoplasm, binds to a calcium channel located on the ER, the calcium channel opens, and calcium diffuses out into the cytosol.



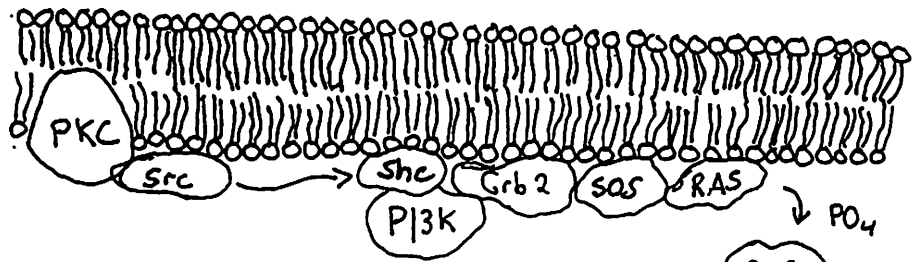
increase in calcium concentration acts as an intracellular messenger which drives vesicles to fuse with the plasma membrane and release their contents. This calcium signal attenuates as calcium is pumped back into the ER lumen.

The second messenger DAG remains associated with the plasma membrane and activates protein kinase C (PKC). PKC remains inactive until increases in cytosolic calcium concentration, from IP_3 , causes conventional PKC to bind to the cytosolic side of the plasma membrane where DAG then activates it. PKC belongs to the serine-threonine kinase family and exists in three forms based on the requirement for activation: conventional, novel, or atypical. Conventional PKC requires DAG and calcium to activate, novel PKC does not require calcium but does require DAG, and atypical PKC requires neither calcium or DAG to activate. The activation of PKC controls the downstream extracellular signal-related kinase (ERK) activity (Figure 2). ERK causes the phosphorylation of transcription factors which leads to the production of mRNA and synthesis of digestive enzymes.

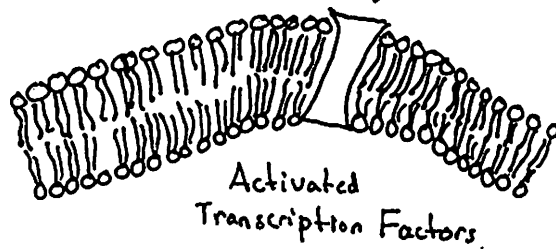
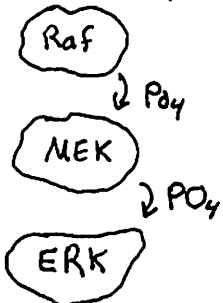
Acinar cells release digestive enzymes into the duodenum after they have undergone modification and then exocytosis. Following the production of mRNA, enzymes are synthesized in the rough endoplasmic reticulum (RER) and then transported to the cis face of the golgi apparatus. These enzymes are inactive precursors known as zymogens. Transport vesicles surrounding the zymogens bud off the RER and fuse with the golgi apparatus membrane facing the nucleus. Once inside the golgi apparatus, zymogens are modified and moved from saccule to saccule by transfer vesicles. Finally, the zymogens bud off the trans golgi membrane as secretory vesicles. These vesicles then fuse with the plasma membrane and eject the

Figure 2. A model for PKC activation of the ERK signalling Cascade. Increases in cytosolic calcium causes conventional PKC to bind to the PM where DAG activates it. PKC activation leads to the downstream activation of the ERK signaling cascade, mRNA production, and synthesis of enzymes.

Extracellular
Space



Cytosol



Nucleus

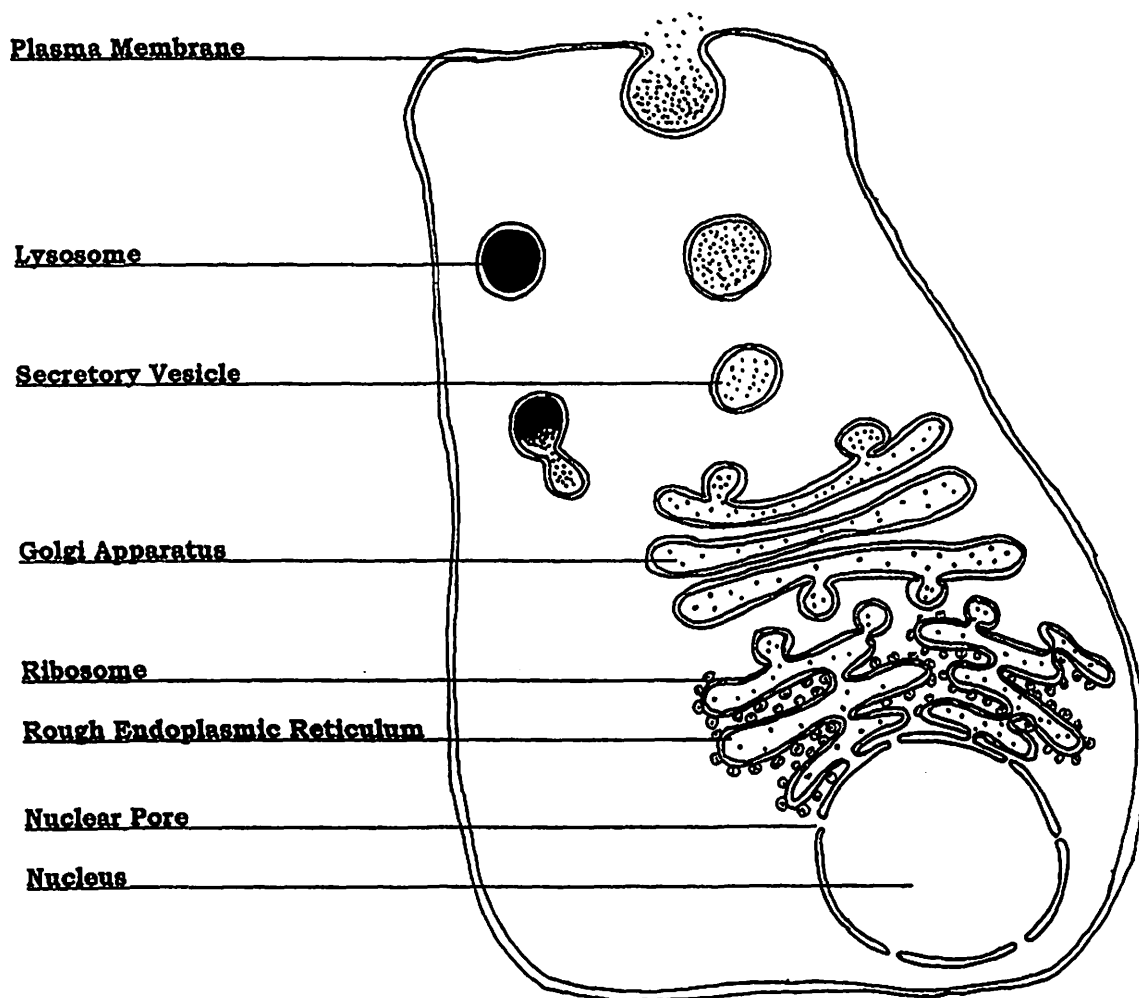
zymogens into the duodenum (Figure 3). The zymogens are then converted into active digestive enzymes by secreted intestinal proteases.

Vesicles released by the golgi apparatus walk or slide along complex cytoskeletal arrangements as they are directed to fuse with the plasma membrane. The cytoskeleton is made up of protein fibers that support cell structure and aid in organelle movement. The cytoskeleton consists of three main groups: microfilaments, microtubules, and intermediate filaments.

Microfilaments are composed of highly conserved actin polymers that aid in secretory vesicle transport. Actin exists in a globular form, g-actin, and in a filamentous form, F-actin. G-actin polymerizes into f-actin with the introduction of an ion signal. Since each actin subunit maintains a polarity, the actin filament is designated with a positive end and a negative end. Polymerization occurs at each end, but elongates faster at the positive end. As the filament lengthens, all ATP is hydrolyzed into ADP and Pi, except the terminal ATP. To further stabilize and prevent depolymerization, actin binding proteins attach to the positive and negative ends of the filament and form a cap.

There are three classes of actin; α , β , and γ actin. The α -actin class has a subtype for skeletal, cardiac, and smooth muscle while β -actin and γ -actin are found in nonmuscular tissue like kidney and liver cells. Actin filaments are arranged in either structural bundles or networks. Bundles arrange filaments in parallel lines, while two types of networks crisscross filaments. The webbed planar network associates with the plasma membrane, whereas a cytosolic network creates a gel-like structure. Both

Figure 3. A model for zymogen production and transportation. Following the production of mRNA, inactive enzymes, or zymogens, are synthesized in the RER. Zymogens are then sent to the golgi apparatus, via transfer vesicles, where they are modified and organized into secretory vesicles. Vesicles are then either shipped to the PM, where they undergo exocytosis and enter the small intestine, or fuse with a lysosome to degrade zymogens.



bundles and networks hold filaments together with a series of specific actin cross-linking proteins.

Cellular movement is achieved with the action of an ATPase myosin enzyme moving along an actin filament. Two types of myosin, myosin I and myosin II, are composed of both a heavy chain and a light chain. The heavy chain has a head domain which binds ATP, a neck domain which associates with the light chain, and a tail domain that contains binding sites to determine myosin activity. Myosin head domains move against actin filaments in combination with ATP hydrolysis. In the absence of bound ATP, myosin binds to actin. ATP then binds to a specialized ATP-binding pocket on myosin II, which causes a conformational change at the actin-binding site. Myosin detaches from actin and hydrolyses ATP. Myosin then reforms into an energized position closer to the positive end of the filament and rebinds with actin. The inorganic phosphate dissociates from myosin causing the energized myosin to rotate and pull actin along with it. In muscle sarcomeres, myosin remains stationary and pulls actin, in nonmuscle systems, actin remains still and myosin walk along the filament.

Cytoskeletal organization and migration have been linked to the Rho family GTPases. Rho family GTPases include 14 members and cycle between GTP and GDP to organize microfilaments. The GTPases are regulated by guanine nucleotide exchange factors and guanine nucleotide dissociation inhibitors that modulate exchange between GTP and GDP (Schwartz and Shattil, 2000). GTPase-activating proteins negatively inhibit GTP activity by promoting GTP hydrolysis. Rho kinases (ROK) stimulate myosin by directly phosphorylating the myosin light chain or by

phosphorylating myosin light chain phosphatases. The phosphatase inactivates and myosin activity increases. The ROK contains a lipid binding site and remains localized to the membrane.

Microtubules, the second of three groups that make up the cytoskeleton, combine globular monomers of the protein tubulin together to form cylindrical tubes. Tubulin, a heterodimeric GTPase, binds GTP to both an α - and β -tubulin subunit, but only the β -tubulin binding site can hydrolyse GTP to GDP. Tubulin monomers produce a protofilament by arranging alternating α - and β -tubulin subunits in longitudinal rows. Thirteen protofilaments combine to form a simple tube called a singlet microtubule. Doublet and triplet microtubules also exist, with one main 13-protofilament microtubule and an additional one or two 10-protofilament microtubule. Microtubules, like actin microfilaments, also have a polarity which distinguishes a positive from a negative end (Kraemer et al., 1998). Microtubules structurally situate themselves so that the positive ends face away from a microtubule-organizing center (MTOC). In animals, the MTOC is a centrosome which can contain a pair of centrioles. The proteins γ -tubulin and pericentrin, necessary to initiate microtubule assembly, are also located within the MTOC.

Microtubules quickly assemble or disassemble their structure in three steps. First, $\alpha\beta$ -tubulin subunits will organize longitudinally into protofilaments. Second, protofilaments associate laterally and form the walls of a singlet microtubule. Then, if $\alpha\beta$ -tubulin subunits reach a specific concentration, microtubules will elongate with the

addition of extra subunits. After β -tubulin attaches to the growing microtubule, the GTP hydrolyses to GDP. If polymerization occurs faster than the hydrolysis of GTP, a cap of GTP-bound subunits will be added to the positive end. Polymerization of microtubules occurs faster at the positive end than at the negative end, so capping is not necessary at the negative end. If the level of $\alpha\beta$ -tubulin subunits decreases below the critical concentration, microtubules will depolymerize. Microtubule-associated proteins (MAPs) further stabilize microtubules by cross-linking to other microtubules, microfilaments, and by preventing depolymerization.

The motor proteins kinesin and dynein transport vesicles along microtubules. Kinesin moves along microtubules in the positive direction whereas dynein moves in the negative direction. Kinesin has two heavy chains which each connect to two light chains. A large dimeric head domain binds to microtubules and ATP hydrolysis generates the motor activity. A tail domain binds to the vesicle being transported. Dynein are large multimeric proteins with two heavy chains forming the head domain and two light chains forming the tail domain. Unlike kinesin, dynein needs the assistance of a microtubules-binding protein, such as dynactin, to mediate transport.

Pancreatic cells have the responsibility of producing enzymes, secreting enzymes, and coordinating the two pathways so that production does not exceed release, or release exceed production. Enzyme production results from gene transcription, mRNA synthesis, and eventual protein translation. Enzyme secretion is stimulated by an increase in intracellular calcium concentration, but how are the two coordinated? The cytoskeleton supplies a network that might be able to communicate

relative status between the two intracellular events. Previous reports have shown that zymogen granules require an intact microtubule system to dock with the cell surface (Kraemer et al., 1998), and that secretion is in some way governed by the actin cytoskeleton (Torgerson and McNiven, 2000). In the present study, using techniques and equipment available to me, I manipulated the cytoskeletal networks with known pharmacological agents while simultaneously measuring the physiological activity of relevant proteins. I determined whether the microfilament and microtubules networks are required for exocytotic secretion and for transmitting the signal for ERK tyrosyl phosphorylation.

MATERIALS AND METHODS

Isolation of Pancreatic Acini

Pancreatic tissue removed from male Sprague-Dawley rats served as the source for isolated pancreatic acini. After light ether anaesthesia, the rats underwent cervical dislocation, a procedure approved by the NIU IACUC committee. The pancreas was then dissected from the rat, minced thoroughly, and placed into 20 ml of Hank's growth media (Medium 199), including; 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 1 mg/1 ml bovine serum albumin (BSA), trypsin inhibitor (0.1 mg/ml), and 0.1 mM phenyl methyl sulfonyl fluoride (PMSF). The pancreatic cells were washed two times in 20 ml of medium. Pancreatic cells were next separated into appropriate experimental groups, re-suspended in 20 ml of medium, and placed into a 37°C shaking water bath prior to experimental treatment.

Chemicals

All necessary reagents, antibodies, agonists, and inhibitors for this project were purchased from various biochemical companies in the United States. Medium 199 (Gibco BRL, Inc. Grand Island, NY), used to wash and incubate pancreatic cells also contained , HEPES (Sigma Chemical Company, St. Louis, MO), BSA (Sigma Chemical Company), the protease inhibitor PMSF (Sigma Chemical Company), and trypsin inhibitor (Gibco BRL, Inc.), which inhibits the serine proteases and trypsin.

Protein A agarose beads were purchased from Boehringer Mannheim and were used to immunoprecipitate proteins following their experimental treatments.

The physiological agonist for pancreatic secretion, cholecystokinin, was purchased from Calbiochem-Novabiochem Corp. LaJolla, CA. The cytoskeleton affecting agent, cytochalasin D (Sigma Chemical Company) inhibits microfilament polymerization. Colchicine (Sigma Chemical Company) prevents polymerization of microtubules. To inhibit myosin I I, 2, 3-butanedione monoxime was used as well as 1-(5 -chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4diazepine (ML-9) a myosin light chain kinase inhibitor. Both were purchased from Sigma Chemical Company. Thapsigargin, purchased at Calbiochem-Novabiochem Corp., was used to inhibit the calcium uptake in cells.

Primary antibodies including polyclonal mouse anti-phosphotyrosine IgG2b, polyclonal rabbit anti-ERK1 IgG, polyclonal rabbit anti-ERK2 IgG, and polyclonal rabbit anti-PI3K IgG were purchased from Santa Cruz Biotechnology, Inc. Horseradish peroxidase labeled anti-rabbit IgG secondary antibody was also purchased from Santa Cruz Biotechnology, Inc.

Treatment of Pancreatic Acini

Pancreatic acinar cells were experimentally treated at 37°C in a shaking water bath. Agonists and/or inhibitors were either massed out or diluted from solubilized concentrated stock solutions and placed directly into the cell suspension. Treated pancreatic cells then underwent a 30 minute assay to determine the rate of exocytotic

secretion or alternatively were placed in an IEC-Centra-8R centrifuge at 50 g for two minutes for centrifugation at room temperature. The resulting pellet was lysed, washed, and subjected to immunoprecipitation of tyrosine-phosphorylated proteins.

Immunoprecipitation

Tyrosine phosphorylated proteins were extracted from the pancreatic acinar lysates by standard immunoprecipitation protocols. Acinar cell fractions were suspended and solubilized by homogenization (glass-Teflon potter) in an immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Nonidet P40, 0.5% deoxycholic acid, 2mM sodium orthovanadate, 1.4 mM TPCK, 0.1 mM leupeptin, 1mg/ml SDS, and 1 mg/ml 0.1 mM trypsin inhibitor and 0.1 mM PMSF. The suspension was clarified by centrifugation at 12,000 g for 10 minutes. The supernatant was removed and incubated with 6 μ l of phosphotyrosine antibody for one hour. Antibody was subsequently harvested by adding 15 μ l of protein-A agarose to each sample. The samples incubated overnight at 4°C , or for a period of six hours, on a rotator to maintain suspension of agarose beads. Protein bound to the agarose-conjugated phosphotyrosine antibody were collected by centrifugation at 12,000 g for 30 seconds. The agarose beads containing the bound antibody and tyrosyl phosphorylated proteins were washed three times in immunoprecipitation buffer for 20 minutes at 4°C. To remove additional nonspecifically bound proteins, the beads were then washed three times in a high salt buffer (500 mM NaCl, 50 mM Tris-Hcl [pH 7.5], 0.1% Nonidet P40) and collected by centrifugation at 12,000 g for 30 seconds. The

beads were then washed three more times in a low salt buffer (50 mM Tris-HCl [pH 7.5], 0.1% Nonidet P40) and again collected by centrifugation at 12,000 g for 30 seconds. Finally, the protein-antibody complexes were dissociated from each other as well as the agarose beads by heating for ten minutes (95°C) in Laemmli reducing buffer (65.2 mM Tris-HCL [pH 6.8], 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 710 mM β -mercaptoethanol). The suspension was then centrifuged at 12,000 g for 30 seconds, and the supernatant (containing all tyrosine phosphorylated proteins) was removed and aliquoted into 20 μ l samples and stored at -80°C.

Protein Assay

Protein was assayed using the Bio-Rad Bradford Protein Assay Kit with bovine serum albumin as a standard. Protein fractions were incubated in 2 ml of a 1:4 dilution of protein dye stock solution for 15 minutes at room temperature. Absorbencies were measured at wavelength of 595 nm with a Beckman DU-60 spectrophotometer.

Amylase Assay

Amylase assays were performed by a modified version of Bernfeld (1955), to determine the extent of secretion from the pancreatic acini during treatment. Maltose was used as a disaccharide standard. Samples, 25 μ l and 50 μ l, were incubated with 75 μ l and 50 μ l of resuspension buffer respectively (0.2% Triton X-100, 50 mM Tris, 100 mM NaCl, and 10 mM CaCl_2) and 100 μ l substrate (1% amylopectin, 40 mM

MOPS, 60 mM NaCl) for 30 minutes in a shaking 37°C waterbath. This reaction was stopped with the addition of 0.2 ml stop-development reagent (1% 3,5-dinitrosalicylic acid, 0.4 N NaOH, 1.06 M sodium potassium tartarate). The resulting fractions were heated for 7 minutes at 95°C in a dry bath. The fractions were cooled and diluted with 2.6 ml of water. Individual absorbencies were read with a Beckman DU-60 spectrophotometer at a wavelength of 530 nm. Amylase release was then plotted as a function of time.

Electrophoresis and Electroblotting

Tyrosine phosphorylated proteins, already solubilized in Laemmli buffer, were removed from -80°C storage and heated (95°C) for 10 minutes. After cooling, equal amounts of protein from each sample were loaded on a discontinuous SDS-polyacrylamide gel (gel composition of 4% acrylamide/bis-acrylamide in the stacking gel and 12% acrylamide/bis-acrylamide in the separating gel). The samples were electrophoresed at 110 volts for 60 - 90 minutes. After electrophoresis, gels and nitrocellulose sheets were equilibrated for 10 minutes in transfer electrode buffer (192 mM glycine, 20 mM Tris, 20% methanol) to remove extraneous salts and detergents from the gel and to uniformly wet the nitrocellulose. Proteins were then electroblotted onto a nitrocellulose membrane for 1 hour at 100 volts at 4°C. The electroblot was performed with the Mini-Trans electroblotting apparatus (Bio-Rad Laboratories).

Western Blotting and Enhanced Chemiluminescence (ECL)

After electroblotting, unoccupied reactive sites on the nitrocellulose sheet were blocked with 10% nonfat dry milk in TTBS (250 mM NaCl, 20 mM Tris [pH 7.6], 0.05% Tween-20, for ERK2, or 500 mM NaCl, 20 mM Tris [pH 7.6], 0.05 Tween-20, for Shc and PI3K) for one hour at room temperature on a rocker. Following blocking, nitrocellulose blots were washed six times by incubating in 20 ml of a TTBS solution for five minutes at room temperature. After washing, nitrocellulose blots were incubated on a rocker overnight at 4°C or for one hour at room temperature in a solution containing 1% non-fat dry milk in specific salt concentration TTBS and a primary antibody. A 1:1000 dilution was used for either stock polyclonal rabbit anti-Shc or ERK2, and a 1:4000 dilution was used for polyclonal rabbit anti-PI3K. The blots were then washed six times for five minutes with TTBS at room temperature on a rocking platform.

Primary antibody binding to proteins was detected using a 1:2000 dilution of stock horseradish peroxidase labeled anti-rabbit IgG. The blot was incubated with the secondary antibody in 1% nonfat dry milk in TTBS for one hour at room temperature on a rocking platform. Following the incubation with the secondary antibody, the blots were washed with TTBS six times for five minutes at room temperature. After the final wash, the blots were exposed to luminol developer solution (equal volumes of cyclic diacylhydrazide luminol and alkaline enhancer). This method relied upon the ability of bound horseradish peroxidase to oxidize the luminol and thereby excite the cyclic diacylhydrazide to a higher energy state. Once excited, the activated compound

decays to a lower energy state and emits a photon of light in the process (wavelength max = 428 nm). The chemiluminescence was detected using blue light-sensitive autoradiographic ECL hyperfilm in a darkroom. This exposure was performed in standard film-holding cassettes for 30 seconds to 30 minutes, depending on which antibody was being used and the amount of the starting sample. The exposed Hyperfilm was then developed and fixed in an automated film developer.

Image Scanning and Qualification

Following western blotting and enhanced chemiluminescence, protein bands on the ECL film were acquired using the computer program Adobe Photoshop 5.0. These scanned protein bands were then quantified using the computer program NIH Image 1.61.

RESULTS

To determine the correlation between cytoskeletal elements and exocytotic secretion, pharmacological agents were added to pancreatic acinar cells to alter the formation of microtubules and microfilaments. Isolated pancreatic acinar cells were treated with the physiological agonist CCK, known to stimulate activation of intermediates in the ERK signaling pathway (Dabrowski et al., 1997). Another group of pancreatic acinar cells from the same preparation were treated with 30 μ M colchicine to disrupt the microtubule network prior to CCK stimulation. Cells were immunoprecipitated with anti-phosphotyrosine antibody and probed using antibodies specific for ERK1, ERK2, or PI3K. Western Blot analysis revealed that CCK induces a 33% rise in ERK1 phosphorylation when compared to a control that received no treatment. Significantly, cells pre-treated with colchicine show virtually no CCK induced ERK1 phosphorylation (Figure 4). These results were verified in other experiments whereby CCK reproducibly increased the level of ERK phosphorylation and when pre-treated with colchicine, prevented ERK phosphorylation (Table 1). In contrast, when PI3K phosphorylation was monitored as opposed to ERK, the results were significantly different, with colchicine causing no inhibition of CCK induced PI3K phosphorylation. This suggests that the role of microtubules is distal to PI3K in the signalling cascade.

To test whether movement along microfilaments is a requirement for signal transduction, two drugs that inhibit the Myosin II ATPase motor protein were employed. The reagent BDM specifically inhibits the contraction of vertebrate muscle and

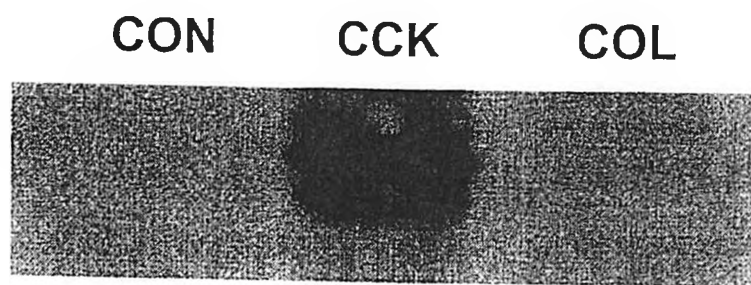


Figure 4. Pancreatic acinar cells treated in colchicine and CCK show no ERK phosphorylation compared to the increased phosphorylation that occurs when cells are only stimulated with CCK.

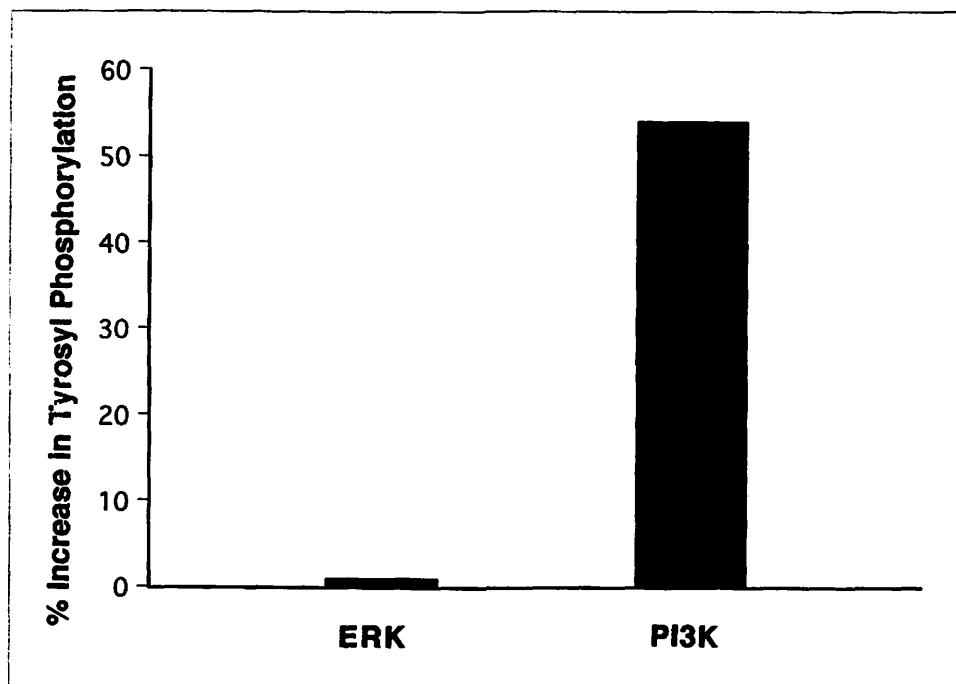


Table 1. The figure shows the change in the level of tyrosyl phosphorylation after stimulation with CCK and treatment with 30 mM colchicine for 30 minutes, in comparison to cells treated only with CCK. ERK showed virtually no phosphorylation, while PI3K showed no change in phosphorylation compared to cells treated only with CCK.

reversibly affects myosin function and influences the intracellular concentration of calcium (Steinberg and McIntosh, 1998). Isolated pancreatic acinar cells were treated with 10 mM BDM for 10 minutes prior to stimulation with CCK. Cells were immunoprecipitated with anti-phosphotyrosine antibody and probed using antibodies specific for ERK2, Shc, or PI3K. Western Blot analysis reveals that CCK stimulated cells alone raise ERK2 phosphorylation by 300% whereas cells treated with thapsigargin, a drug that selectively increases intracellular calcium concentration, caused a 110% increase in ERK2 phosphorylation. Cells treated with BDM and CCK increase ERK2 phosphorylation by 45%, in the presence of thapsigargin and compared to CCK alone. Analysis specific for Shc shows a 41% phosphorylation with CCK, an increase of an additional 42% in phosphorylation when cells were pre-treated with BDM, CCK, and thapsigargin, whereas thapsigargin alone promoted ERK phosphorylation to levels similar to that of CCK treatment. Likewise PI3K phosphorylation also increased in cells when pre-treated with BDM, compared to CCK and thapsigargin alone (Table 2).

To determine whether inhibition of myosin motor proteins had a proportional effect on the exocytotic branch of the signally system, the rate of pancreatic amylase release was measured. Isolated cells were either stimulated with CCK or treated with 10 mM BDM and then stimulated with CCK. Total amylase release was measured over the course of 30 minutes and compared to a sample of untreated cells. The untreated cells released 10% of the total amylase over the course of 30 minutes, which is within normal experimental ranges. Cells stimulated with CCK responded with a significant increase in secretion rate releasing 35% of the total amylase over the

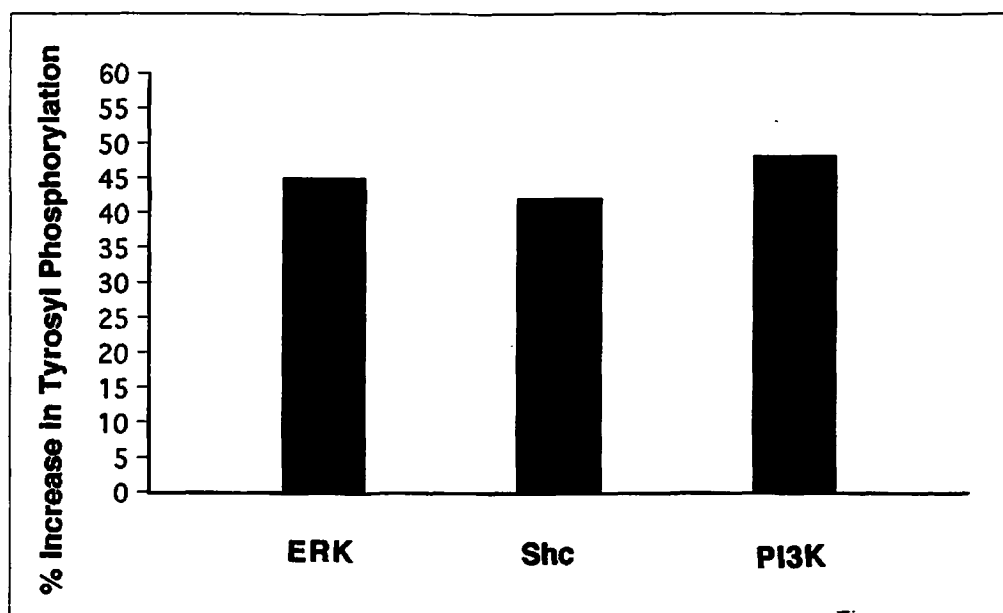


Table 2. The effect of BDM on the tyrosyl phosphorylation of ERK, Shc, and PI3K, in the presence of thapsigargin and compared to cells only stimulated with CCK. Cells pre-treated with BDM and CCK showed a 45% phosphorylation of ERK, a 42% phosphorylation of Shc, and a 48% phosphorylation of PI3K. These were increases in tyrosyl phosphorylation compared to cells treated only with CCK.

course of 30 minutes. However, cells treated with BDM prior to stimulation with CCK produced only a 10% release of total amylase, which is almost identical to the amylase release of non-treated cells (Figure 5). These results suggest the activity of myosin motor proteins are required for exocytosis, but down-regulates the ERK transcription branch of cell coordination.

To further investigate whether movement along microfilaments is necessary for signal transduction, myosin light chain kinase was inhibited by treating cells for 15 minutes with 30 μ M ML-9. An initial experiment first treated isolated cells with CCK, and then isolated cells with both CCK and ML-9. Cells were immunoprecipitated with anti-phosphotyrosine antibody and probed using antibodies specific for ERK2 and PI3K. Cells stimulated with CCK alone showed a dramatic 431% increase in ERK2 phosphorylation compared to cells that underwent no treatment. Cells treated with both CCK and ML-9 showed a 13% increase in ERK2 phosphorylation compared to cells stimulated only with CCK. Likewise, ML-9 increased CCK induced PI3K phosphorylation by 26% compared to cells stimulated only with CCK (Table 3). A second experiment added thapsigargin to CCK stimulated cells treated with ML-9. Cells stimulated with CCK alone have a 749% ERK2 phosphorylation compared to untreated cells. Cells treated with ML-9, CCK, and thapsigargin decrease ERK2 phosphorylation by 16% over CCK stimulated cells. Thapsigargin alone increased only 8.7% compared to untreated cells. Analysis specific for PI3K showed a 30% increase in PI3K phosphorylation with CCK compared to untreated cells, a 2% decrease in phosphorylation with ML-9, CCK, and thapsigargin (compared to CCK alone), and a 19% decrease in PI3K phosphorylation with thapsigargin alone

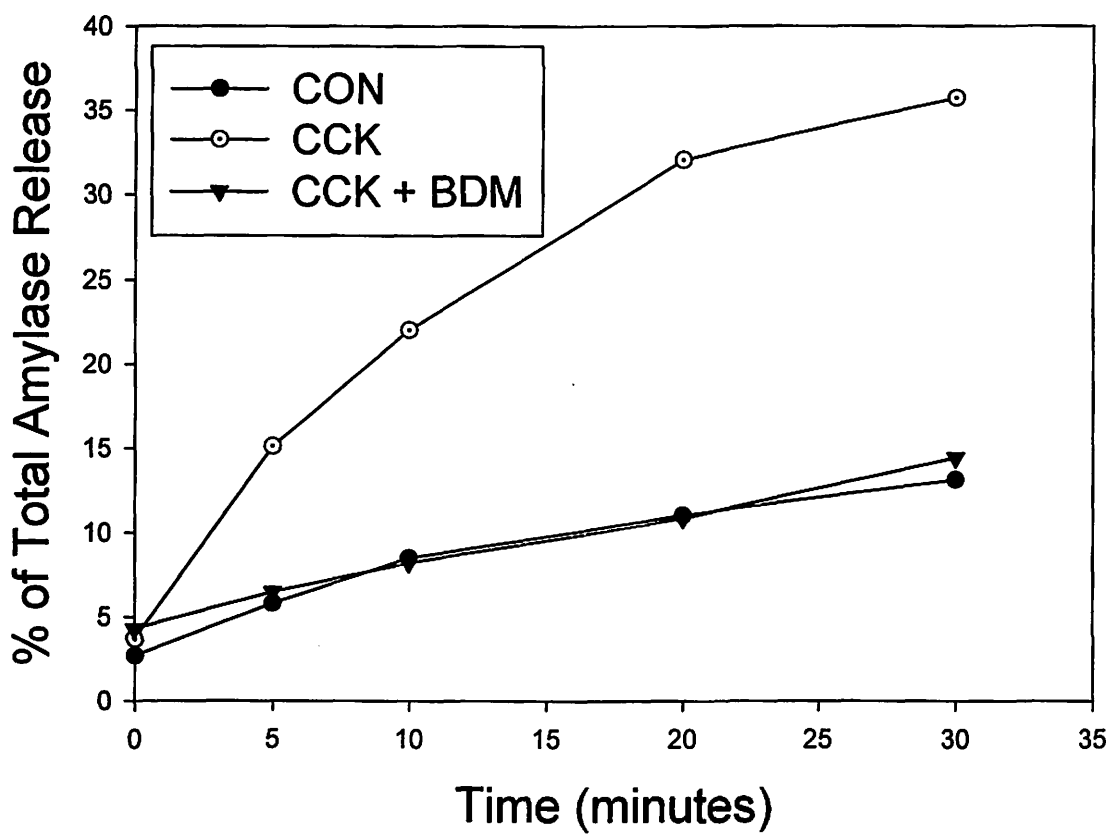


Figure 5. Pancreatic acinar cells treated with both BDM and CCK for 30 minutes show virtually no amylase secretion compared to cells treated with CCK, which show a 35% increase compared to cells treated with nothing.

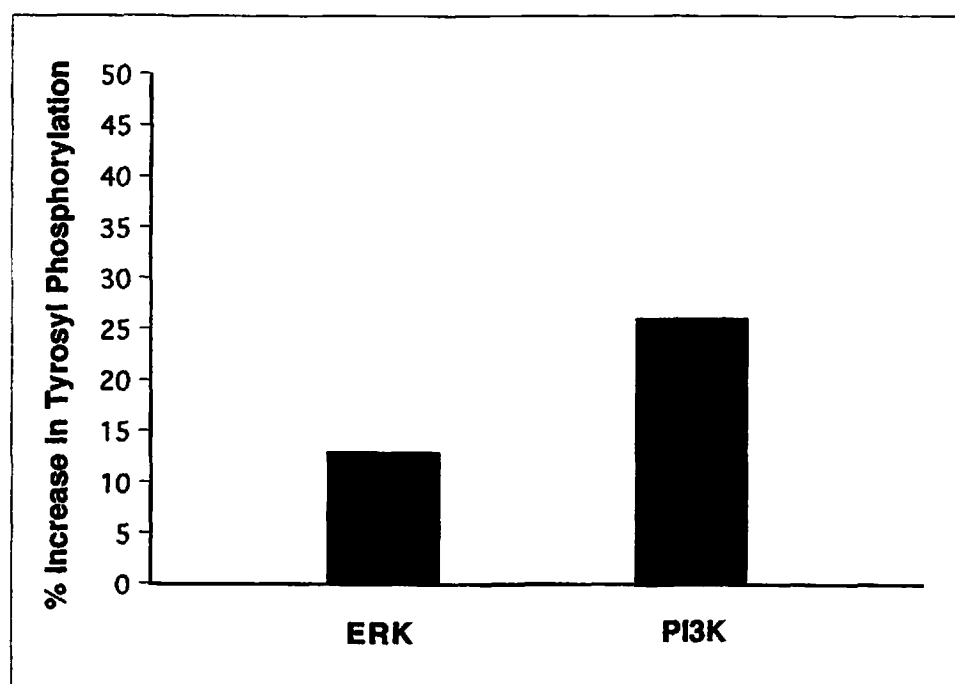


Table 3. The effect of ML-9 on the phosphorylation of ERK and PI3K, compared to cells only stimulated with CCK. Cells treated with ML-9 and CCK showed a 13% phosphorylation of ERK and a 26% phosphorylation of PI3K. These were increases in tyrosyl phosphorylation compared to cells treated only with CCK.

compared to untreated cells (Table 4).

A final experiment was done to determine whether or not actin filaments are necessary for signal transduction. Acinar cells were treated with 30 μ M cytochalasin D for 40 minutes and zymogen granules were isolated. Cytochalasin D binds to the end of actin filaments preventing monomer polymerization. Cells were immunoprecipitated with anti-phosphotyrosine antibody and probed using antibodies specific for PI3K. Compared to cells that were not treated with cytochalasin D, PI3K had a 75% decrease in phosphorylation. This suggests that microfilament need to polymerize to ensure successful signal transduction and PI3K phosphorylation.

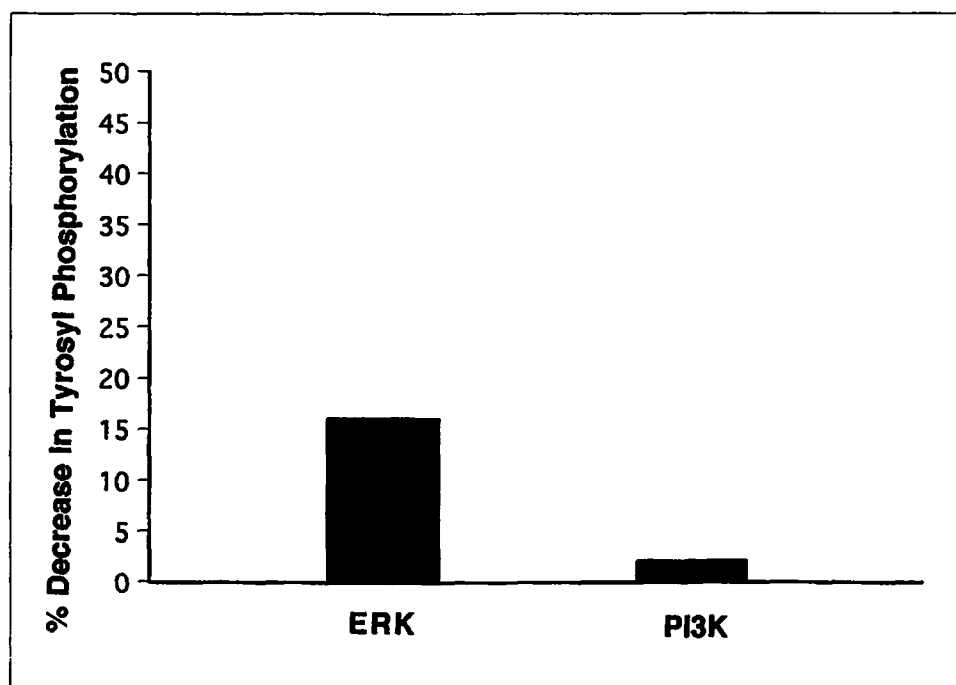


Table 4. The effect of ML-9 and thapsigargin on the tyrosyl phosphorylation of ERK and PI3K, compared to cells only stimulated with CCK. Cells treated with ML-9, CCK, and thapsigargin showed a 16% phosphorylation of ERK and a 2% phosphorylation of PI3K. These were decreases in tyrosyl phosphorylation compared to cells treated only with CCK.

DISCUSSION

The pancreas must respond to CCK stimulation by producing digestive enzymes while simultaneously initiating enzyme release into the small intestine. After systematically altering the cytoskeletal network within pancreatic cells, physiological coordination showed a disruption between enzyme production and secretion. The potential for denovo enzyme production was measured based on the activation of signaling enzymes that lead to gene transcription. The enzymes in the study were activated by phosphorylation. Phosphorylation entails covalent attachment of phosphate (PO_4^-) to a tyrosine residue on the enzyme. This addition of negatively charged group alters the protein structure resulting in the activation of the catalytic site. Initial experiments preventing microtubule polymerization resulted in a dramatic decrease in extracellular signal-related kinase (ERK) phosphorylation. ERKs are protein serine/threonine kinases that are activated by cell surface receptors. ERKs function in signal cascade pathways that lead to the expression of transcription factors which direct mRNA synthesis (Duan and Williams, 1994). To maintain signal transduction, the results conclusively demonstrated that microtubules are necessary for ERK phosphorylation, which leads to mRNA production and eventual enzyme synthesis. However, the results suggest that microtubules are not required for the entire signal transduction cascade. Disrupting the microtubules did not alter PI3K phosphorylation compared to normally treated cells. PI3K kinase resides on the plasma membrane and is proximal to ERK on the cascade. Phosphatidylinositol 3-kinase (PI3K), belongs to a family of enzymes that are heterodimers and consist of a

regulatory subunit and a catalytic subunit. PI3K appears to mediate aspects of protein trafficking in cells (Lopez-Illasaca, 1998). Without a stable microtubule network, cell coordination, as well as cellular structure, no longer sustain normal physiological signal conduction, preventing both enzyme synthesis and release.

Cells that underwent microfilament inhibition, however, resulted in increased signal transduction, but decreased amylase secretion. Instead of simply preventing polymerization of microfilaments, cells were treated with BDM to inhibit myosin ATPase activity. As a result, the signal cascade continued to phosphorylate PI3K and ERK, but vesicles were not being released at the plasma membrane. Myosin ATPase activity is not required for ERK or PI3K phosphorylation, which leads to enzyme production, but is necessary to transport vesicles containing enzymes to the plasma membrane for secretion. When myosin ATPase activity was blocked with BDM in the presence of increased intracellular calcium concentration, ERK and PI3K showed significant increased phosphorylation over CCK stimulated cells.

To verify these results, motor proteins were obstructed from moving along actin filaments by treating cells with ML-9, an inhibitor of myosin light chain kinase. Both ERK and PI3K maintained levels of phosphorylation within cells treated with ML-9. Myosin motor proteins are therefore not directly required for phosphorylation of signaling enzymes within signal transduction cascade, but theoretically are required for vesicle transport to the plasma membrane. Further experiments need to be performed that measure amylase release of a cell treated with ML-9 to confirm a decrease in cell secretion.

ML-9 treatment of pancreatic acinar cells with high intracellular calcium caused

ERK and PI3K phosphorylation to decrease compared to cells treated with CCK or high calcium alone. Although mechanistically unclear, increased calcium prevents cell phosphorylation in cells that have not myosin motor activity. Therefore, both ML-9 and BDM, agents that inhibit myosin motor proteins via different mechanisms, cause a significant decrease in ERK and PI3K when the calcium secreting signal is high, suggesting a feedback relationship.

A final experiment was conducted that prevented actin filament polymerization. Similar to cells that had microtubule polymerization inhibited with colchicine, cells apparently cannot function appropriately when either element of the cytoskeletal network has been depolymerized. Cells treated with cytochalasin D however showed decreased PI3K phosphorylation compared to untreated cells. Whereas colchicine induces microtubule depolymerization but did not alter PI3K phosphorylation. In the case of microfilaments, PI3K requires stable microfilament polymerization to be activated. This is consistent with reports that the microfilament cytoskeleton can promote linkage of numerous signalling molecules into a coordinated complex (Torgerson and McNiven, 2000). The results also suggest that ERK is distal to this complex.

In response to the disconnection between enzyme production and secretion, a form of feedback control possibly governs the two interrelated events under normal conditions. The present results would suggest that when secretion increases, a signal feeds back into the signaling cascade which subsequently increases enzyme production. Additionally, if cell secretion falls below a certain level, a feedback mechanism further increases phosphorylation of signal cascade enzymes. The results

demonstrated that a dynamic cytoskeleton was required for activation of the ERK signalling cascade and synthesis of additional digestive enzymes. The results further demonstrate that myosin motor protein activity is necessary to down regulate ERK activity in the presence of calcium. In these experiments, disrupting the cytoskeleton decreased secretion which hypothetically communicated the feedback signal to increase ERK phosphorylation and the increase of enzyme production. Nevertheless, further examination of cell physiology needs to occur to precisely identify the feedback signal that leads to coordination of pancreatic activity.

REFERENCES

- Dabrowski, A., G. Groblewski, C. Schafer, K. Guan., and J. Williams. 1997. Cholecystokinin and EGF activate a MAPK cascade by different mechanisms in rat pancreatic acinar cells. *Amer. J. Physiol. Soc., C*: 1472-1479.
- Duan, R. And J. Williams. 1994. Cholecystokinin rapidly activates mitogen-activated protein kinases in rat pancreatic acini. *Amer. Physiol. Soc.*, 267: G401-G408.
- Kraemer, J., F. Schmitz, and D. Drenckhahn. 1999. Cytoplasmic dynein and dynactin as likely candidates for microtubule-dependent apical targeting of pancreatic zymogen granules. *Eur. J. Cell Biol.*, 78: 265-277.
- Lopez-Illasaca, M. 1998. Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem. Pharmacol.*, 56: 269-277.
- Schwartz, M.A. And S.J. Shattil. 2000. Signaling networks linking integrins and Rho family GTPases. *Inst. Biol. Sciences*, Aug: 288-341.
- Torgerson, R.R. and M.A. McNiven. 2000. Agonist-Induced changes in cell shape during regulated secretion in rat pancreatic acini. *J.Cell. Physiol*, 182: 438-447.